The Roles of EXT-Like Proteins in Heparan Sulphate Biosynthesis

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ABSTRACT

Heparan sulphate (HS) proteoglycans, composed of a core protein and one or more negatively charged HS chains, are a major component of extracellular matrices and are located on the plasma membrane of all animal cells. The HS polysaccharide is synthesized by alternating addition of glucuronic acid (GlcA) and N-acetylglycosamine (GlcNAc) from corresponding UDP-sugar precursors. Due to subsequent modifications, including epimerization of GlcA to iduronic acid and sulphation the resultant polysaccharide is variously sulphate-substituted at different positions. Polymerization of HS chains is believed to be catalyzed by the EXT family of proteins. In humans, the EXT family consists of five members: EXT1, EXT2, EXTL1, EXTL2 and EXTL3. All members of exostoses family harbour *in vitro* glycosyltransferase activities related to HS chain elongation. EXT1-EXT2 form a heter-oligomeric complex involved in the chain elongation step in HS biosynthesis, but the biological roles of EXTLs are less clearly defined. The aim of the studies presented in this thesis was to investigate the roles of the three EXTLs in heparan sulphate biosynthesis. Down regulation of EXTL2 or EXTL3 expression in a human cell line resulted in increased HS chain length. Also overexpression of EXTL1 or EXTL3 resulted in increased chain length. In contrast, overexpression of EXTL2 resulted in shorter HS chains. The results indicate that these enzymes influence HS synthesis and that their expression levels control HS chain length. In addition, all three EXTL proteins were able to catalyze the addition of a single GlcNAc to [GlcA-GlcNAc]$_n$ oligosaccharide acceptors, but did not show any detectable transfer of GlcA to GlcNAc[GlcA-GlcNAc]$_n$ oligosaccharide acceptors.

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SCIENTIFIC ENVIRONMENT

The experimental work presented in this thesis was carried out at the Department of Biomedicine, University of Bergen, Norway. The work was performed in the Matrix Biology group, under supervision of Professor Marion Kusche- Gullberg, as my main supervisor and Professor Donald Gullberg as my cosupervisor.

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LIST OF PAPERS

The present thesis is based on following two papers:

Paper I: The role of EXTL2 in heparan sulphate chain elongation
Tabasum Imran and Marion Kusche-Gullberg
Manuscript

Paper II: Effect of over expression of EXTL1 and EXTL3 on heparan sulphate chain length
Tabasum Imran, Ning Lu and Marion Kusche-Gullberg
Manuscript

ABBREVIATIONS

2OST	2-O-sulfotransferase
3OST	3-O-sulfotransferase
6OST	6-O-sulfotransferase
botv	brother of tout-velu
CS	Chondroitin sulphate
DS	Dermatan sulphate
ECM	Extracellular matrix
EXT	Exostosin
EXTL	Exostosin like
FGF	Fibroblast growth factor
GAG	Glycosaminoglycan
Gal	Galactose
GlcA	Glucuronic acid
GlcNAc	N-acetylglucosamine
GPI	Glycosylphosphatidylinositol
Gly	Glycine
HS	Heparan sulphate
HSPG	Heparan sulphate proteoglycan
IdoA	Iduronic acid
MO	Multiple Osteochondromas
NAc	N-acetylated
NDST	N-deacetylase/N-sulfotransferase
NS	N-sulphated
OST	O-sulfotransferase
PAPS	3'-phosphoadenosine-5'-phospho sulphate
PG	Proteoglycan
Ser	Serine
siRNA	small interfering RNA
sotv	sister of tout-velu
sulf	6-O-endosulfatases
TM	Transmembrane
ttv	tout-velu
UDP	Uridine-5-diphosphate
Xyl	Xylose

INTRODUCTION

Carbohydrates or saccharides are essential components of all living organisms. The study of the biosynthesis, structure and function of carbohydrate is referred as glycobiology (Rademacher at al., 1988, Rademacher, 1998). Carbohydrates can occur as single units (monosaccharides) or as larger structures composed of 2-10 (oligosaccharides) or more (polysaccharides, glycans) sugar residues. Glycoconjugates consists of one or more sugar units covalently attached to a protein or a lipid. Most cells have sugar coating on their surface and sugar chains can make an important portion of the glycoconjugates. Cell surfaces associated glycoconjugates are mostly attached to the plasma membranes through their protein or lipid moieties. Carbohydrates play an important role in protein modifications and in energy metabolism and can effect interactions between cells and their surroundings. Carbohydrates have complex physical and chemical characters and are often heterogeneous both in size and composition (Varki et al., 1999, Schuman et al., 2007). In contrast to DNA, RNA and proteins, carbohydrate sequences are not determined by reading of a template. Instead carbohydrates are synthesized based upon substrate specificity of different glycosyltransferases (Park et al., 2000, Guimond et al., 2001, Jones and Vogt, 2001).

PROTEOGLYCANS AND GLYCOSAMINOGLYCANS

Proteoglycans (PGs) are complex molecules that are composed of a protein core with covalently attached long unbranched polysaccharide chains that are called glycosaminoglycans (GAGs) (Esko and Selleck, 2002). PGs are classified on the basis of the polysaccharide chains or the core proteins. GAGs are linear, and negatively charged polysaccharides and are generally grouped into four categories: (1) hyaluronic acid or hyaluronan, which occurs as free GAG chains (*i.e.* not linked to core protein), (2) heparin and heparan sulphate (HS), (3) chondroitin sulphate (CS)/dermatan sulphate (DS) and (4) keratan sulphate (Figure 1). They are composed

of repeating disaccharide units with an N-acetylhexosamine (N-acetylglucosamine or N-acetylgalactosamine) as one of the sugar unit. The alternating sugar is glucuronic acid (GlcA) with the exception of keratan sulphate that instead contains galactose (Lindahl et al., 1998, Sugahara and Kitagawa 2000, Turnbull et al., 2001, Esko and Selleck, 2002).

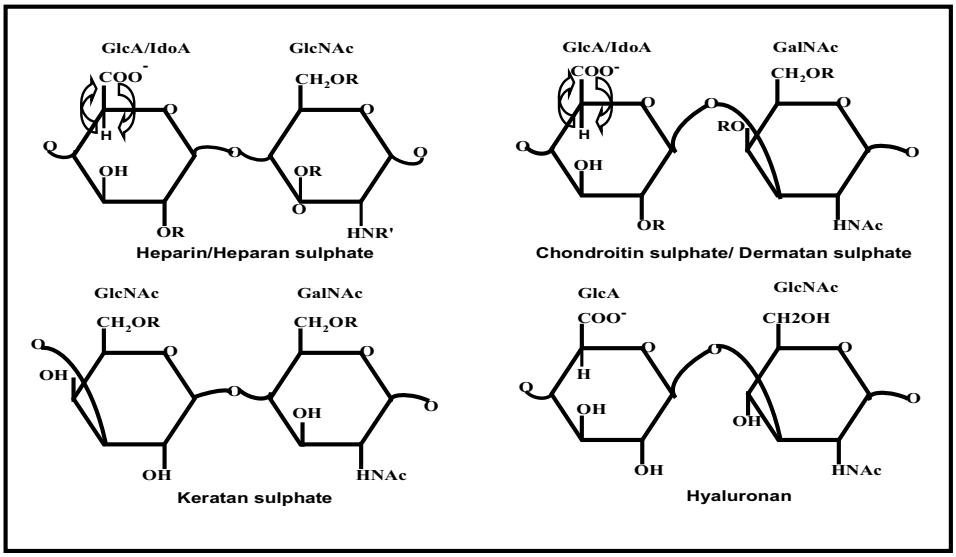


Figure 1. Disaccharide repeating structure of different glycosaminoglycans.

Possible modifications of the basic disaccharide structures are indicated by R=H or SO_3^- and *R'*=$COCH_3$, SO_3^-or H. The glucuronic acid in heparin/HS and CS can be epimerised into iduronic acid (indicated by arrows).

HEPARAN SULPHATE/ HEPARIN PROTEOGLYCANS

Heparin and HS are two closely related GAGs that are synthesized as part of a PG (Kjellén and Lindahl, 1991). Heparin was discovered by Mclean in 1916 and was found, by mere chance, to inhibit blood coagulation (McLean, 1916). Much later, 1968, evidence was presented that indicated that the anticoagulant activity of heparin was mediated by the plasma protease inhibitor antithrombin (Abildgaard, 1968) but actual binding of heparin to antithrombin was first shown in the 1970s by Damus and

Rosenberg (Damus et al., 1973). HS was originally discovered as an impurity of heparin preparations (Linker at al., 1958) but it has since then become evident that the HS chains of a HSPG are involved in many different biological processes. In contrast to heparin that occurs exclusively in connective-tissue type mast cells, HS is produced by all multicellular organisms from ancient cnidarians to modern mammals, and occurs at the cell surface, and in the extracellular matrix (ECM) (Lindahl 2007, Lindahl and Li, 2009). Due to the structural heterogeneity of the HS chains, HS cannot be considered as a single molecule but rather a family of related molecules and heparin can thus be regarded as a highly sulphated variant of HS (Li and Vlodavski 2009, Lindahl and Li, 2009). HS chain structure is believed to be cell type but not core protein specific, so that all HS chains synthesized by a given cell have similar structures although they may be linked to different core proteins (Forsberg and Kjellén, 2001). Several different PG proteins carry HS chains (Figure 2, Table 1). Two of the HS families, the syndecans and glypicans involve HSPGs that are located at plasma membrane of cells (reviewed in Bernfield et al., 1999, Kirn-Safran et al., 2009). Another group of HSPGs are the secreted forms, including perlecan, agrin, and collagen XVIII. Serglycin, is an intracellular PG found in haemopietic cells and in endothelial cells that, dependent on the cellular origin, carries heparin and/or CS chains (Kolset et al., 2004).

Syndecans

Syndecans are found at cell surface and constitute a family with four members in mammals and probably in all vertebrates as well. *C. elegans* and *D. melanogaster* has only one syndecan (Minniti et al., 2004, Steigemann et al., 2004), while zebrafish has three syndecans (syndecan-2, -3, and -4) (Chakravarti and Adams, 2006). Most cells and tissues express at least one member of this protein family and many express multiple syndecans (Couchman 2003, Yoneda and Couchman, 2003). Vertebrate syndecan family are type 1 glycoproteins. Each syndecan has three major domains, a large extracellular ectodomain, a short cytoplasmic domain, and a single highly

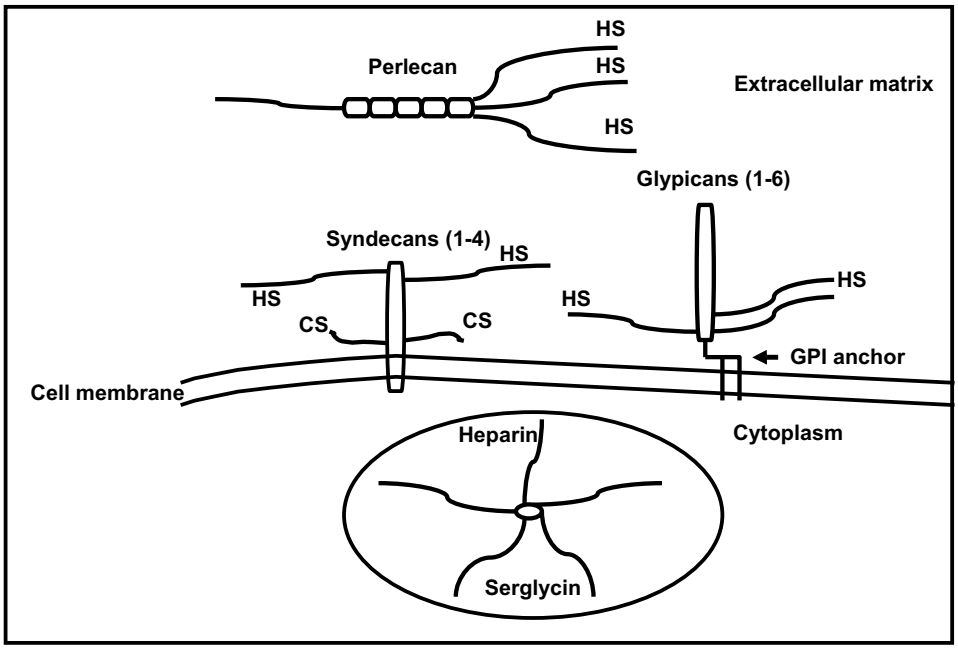


Figure 2. Schematic overview of the main heparin/heparan sulphate proteoglycans.

Syndecans and glypicans are both cell membrane associated PGs. Syndecans are transmembrane proteins that bears HS chains distal from plasma membrane. Some syndecans also carry CS chains close to the plasma membrane. Glypicans are attached to cell surface membrane via a GPI anchor and HS chains are located near the plasma membrane. Perlecan is an example of an extracellular PG. Serglycin, stored intracellular in the secretory granules of connective tissue mast cells, is decorated with heparin chains.

conserved transmembrane domain (reviewed in Couchman 2003, Xian et al., 2009). The extracellular ectodomains are variable in length and sequence between syndecan members and contain conserved GAG attachment sites. HS is the principal GAG present in all four syndecans and is usually found close to the N-terminus of syndecan extracellular domain. Syndecan-1 and syndecan-3 may also carry CS chains close to the plasma membrane. The transmembrane domain is important for syndecan dimer formation. Dimerization is essential for syndecan-4 dependent activation of protein kinase Cα and signalling (Oh et al., 1997, Woods and Couchman 1998, Couchman,

Table 1. Examples of heparan sulphate proteoglycans

Proteoglycans	Core protein	Number of glycosaminoglycane	Tissues distribution
Syndecans 1-4	31-45	1-3 CS 1-2 HS	Membrane bound, epithelial cells, cartilage and neuron
Glypicans 1-6	60	1-3 HS	Membrane bound, osteoblasts, skin, spinal cord, glomeruli
Perlecan	400	1-4 HS/CS	Secreted, basement membrane and cartilage
Serglycin	10-19	10-15 Heparin/CS	Intracellular granules, mast cells
Agrin	200	1-3 HS	Secreted, neuromuscular junction
Betaglycan	110	1 HS 1 CS	Membrane bound fibroblasts
Collagen XVIII	147	2-3 HS	Secreted, basement membrane

Varki et al., 1999. Essentials of Glycobiology, second edition

2003). The small cytoplasmic domain has two conserved domains, C1 and C2, which are hall-marks of the syndecans. Adjacent to the plasma membrane is the C1 domain that mediates binding to cytoskeletal proteins (e.g. ezrin) (Granes et al., 2000, Granes et al., 2003). The C2 domain that is more distal to the plasma membrane has binding sites for PDZ (postsynaptic density 95, disc large ZO-1) domain-containing proteins such as syntenin (Grootjans et al., 1997), synbidin (Ethell et al., 2000) and synectin (Gao et al., 2000). PDZ proteins have role as scaffolding proteins and in vesicle trafficking (Zimmermann et al., 2002, Zimmermann et al., 2005). Between C1 and C2 is the variable region that is unique to each syndecan and is conserved across the species. It is believed that most of the functional difference among syndecans is determined by the variable regions (Oh et al., 1997). The variable region of syndecan-4 binds to phosphatidylinositol 4,5-biphosphate 2 (PIP2) and activates protein kinase Cα and is involved in focal adhesion assembly (Oh et al., 1997, Lim et al., 2003). Syndecans are widely expressed throughout the development as well as in adulthood, and individual family members show cell-tissue and developmental-specific

expression (Yoneda and Couchman, 2003). Syndecan-1 is predominantly expressed in mesenchymal and epithelial cells and is involved in angiogenesis, wound healing and leukocyte-endothelial interactions (Woods and Couchman 2001, Couchman et al., 2001). Syndecan-2 is expressed in vasculature, particularly during embryogenesis (Chen et al., 2004). Reduction of syndecan-2 levels in mouse brain microvascular results in decreased capillary tube formation (Fears et al., 2006). In *Xenopus laevis*, syndecan-2 expression in the ectoderm is required for normal left-right axis that is necessary for asymmetric placement of the developing heart (Kramer and Yost, 2002). Syndecan-3 is mainly found in neural tissue and cartilage (Alexander et al., 2000). Syndecan-3 knock mice are resistant to diet-induced obesity (Strader et al., 2004) and show neural migration defects in brain (Hienola et al., 2006). Syndecan-4 is widely expressed through all stages of embryonic development but has restricted expression in adult (Woods and Couchman 1994, Woods and Couchman 2001, Zhang et al., 2003). Syndecan-4 deficient mice show delayed wound healing and impaired angiogenesis (Echtermeyer et al., 2001).

Syndecans can be shed from the cell surface by the action of proteases (Endo et al., 2003). Shedding of syndecan-1 ectodomain has been shown to regulate cell movements suggesting that the shed ectodomain may convey motility signals distinct from the cell surface bound form. Upon release of the ectodomain, the intracellular domain can signal independently or may be degraded. In wound healing, increased levels of syndecan-1 and syndecan-4 are shed into wound fluids (Tumova et al., 2000, Endo et al., 2003).

Glypicans

HSPGs also attach to the cell surface by a family of glycosylphosphatidylinositol (GPI) anchored core proteins called glypicans (reviewed in Bernfield et al., 1999, Kramer and Yost 2003, Filmus et al., 2008). Their protein core consists of a N-terminal signal peptide, a cystein rich globular ectodomain, and a hydrophobic cytoplasmic domain that is involved in the formation of the GPI anchor. The

cytoplasmic domain contains three to four attachment sites for HS close to the cell surface. Six glypicans have been characterized in human and mouse (glypican 1-6), two in *Nematostella vectensis*, five in zebrafish and two in *D. melanogaster* (De Cat and David 2001, Filmus et al., 2008). During embryonic development glypican-1 is expressed in skeletal muscles, osteoblasts, skin, and the glomeruli (Litwack et al., 1998). In the adult, glypican-1 is expressed in most tissues (David et al., 1990, Litwack et al., 1998). Glypican-2 occurs specifically in developing brain, especially located to the spinal cord and dorsal root ganglia and appears not to be present in adults (Ivins et al., 1997). Glypican-3, -4, -5, and -6 is found in most tissues during embryonic development while their expression is more restricted in adult animals. Glypican-3 is so far the only cell surface HSPG core protein that has been linked to a human disease. The X-linked human overgrowth syndrome, Simpson-Golabi-Behmel disorder, is caused by mutation in glypican-3 gene (Pilia et al., 1996). This disease is characterized by tissue and organ overgrowth, macroglossia, congenital defects and an increase risk for developing various tumours. The glypican-3 deficient mice resemble several of the clinical findings of the human patients such as development overgrowth, dysplastic kidneys and abnormal lung development (Pellegrini et al., 1998, Filmus, 2001).

Glypicans can be proteolytically cleaved near the centre of the core protein by a furin-like convertase (De Cat et al., 2003, Fransson et al., 2004). The GPI anchor can be cleaved by phospholipases or by proteases, which causes shedding of glypican ectodomain from the cell surface (David et al., 1990, Lugemwa and Esko 1991, Traister et al., 2008). In *D. melanogaster* the shed glypicans play a role in morphogen gradient formation (Fujise et al., 2003, Han et al., 2004).

Extracellular matrix heparan sulphate proteoglycans

The major secreted HSPG species are perlecan (Knox and Whitelock, 2006), collagen XVIII and agrin present in the ECM (Iozzo, 2005). Perlecan core protein contains four potential GAG attachment sites for HS and CS, whereas agrin has three GAG

attachment sites for HS (Bezakova and Ruegg 2003, Knox and Whitelock, 2006). Agrin is primary considered a neural PG and recognised as a key player in formation, maintenance and regeneration of neuromuscular junction (Prydz and Dalen 2000, Bezakova and Ruegg, 2003). Collagen XVIII expressed in the basement membrane of mouse and human tissues carries HS chains (O´ Reilly et al., 1997, Iozzo, 2005). Endostatin, the C-terminal of collagen XVIII, contains anti-angiogenic properties and treatment with endostatin can reduce tumour growth by interfering with tumour angiogenesis (O´Reilly et al., 1997, Abdollahi et al., 2004).

Serglycin

Serglycin is found in haemopietic cells and in endothelial cells. In the secretory granules of connective tissue mast cells, serglycin is substituted with the highly negatively charged heparin (Kolset et al., 2004). Serglycin knock out mice develop normally, but the mast cells have fewer secretory granules and reduced amounts of histamine (Abrink et al., 2004, Niemann et al., 2007), indicating that serglycin is involved in generation of storage granules in mast cells.

BIOSYNTHESIS OF HEPARAN SULPHATE

HS biosynthesis is carried out by a complex enzymatic machinery involving many different enzymes. Most GAGs attached to a protein share a common multistep biosynthetic process (reviewed in Lindahl et al., 1998, Esko and Lindahl 2001, Esko and Selleck, 2002). This process involves a series of glycosyltransferases in the Golgi compartment transferring nucleotide sugars to oligo- or polysaccharides or proteins and sulfotransferases utilizing 3'-phosphoadenosine-5'-phosphosulphate (PAPS) as sulphate donor. PAPS similar to nucleotide sugars is synthesized in the cytoplasm and imported into endoplasmic reticulum by specific transporter proteins (Hirschberg et al., 1998, Dick et al., 2008). HS chains synthesis is initiated by the formation of a

tetrasaccharide linker attached to a selected serine residue in the core protein. After that, chain elongation proceeds by sequential addition of GlcA and N-acetyl glucosamine (GlcNAc) residues. During chain elongation, HS chains undergo extensive modifications including sulphation and epimerization, resulting in variety of structurally diverse HS chains (Lindahl et al., 1998, Bernfield et al., 1999, Esko and Selleck, 2002) (Figure 3). The regulation of this process, as required to generate specific HS structures, is poorly understood, but apparently involves arrays of enzyme isoforms that differ with regard to substrate specificities and kinetic properties.

Formation of linkage region and heparan sulphate chain assembly

Linkage region formation is initiated by the transfer of xylose from uridine-5-diphoshate (UDP)-xylose to a specific serine residue in the core protein (Zhang and Esko 1995, Sugahara and Kitagawa, 2002). This reaction is followed by addition of two galactose residues and the so-called GAG-protein tetrasaccharide linkage is completed by addition of GlcA (Sugahara and Kitagawa 2000, Bishop et al., 2007). These reactions are catalysed by specific glycosyltransferases. The tetrasaccharide linkage is common for PGs carrying glucosaminoglycans (heparin and HS) and galactosaminoglycans (CS and DS) (Esko and Selleck, 2002). Addition of the subsequent sugar unit to the tetrasaccharide linker determines the type of GAG chain that will be synthesized (Esko and Zhang 1996, Pyrdz and Dallen, 2000). If a GlcNAc is added HS or heparin is synthesised but if instead, N-acetylgalactosamine is added, it leads to the synthesis of CS/DS. The exact mechanism that determines whether HS or CS/DS will be synthesized is not known but it is generally believed that it depends on enzyme recognition of the PG polypeptide (Esko and Zhang 1996, Chen and Lander, 2001). Acidic as well as hydrophobic repetitive ser-gly sequence on the core protein facilitates GlcNAc attachment and the formation of HS/heparin (Lugemwa and Esko 1991, Zhang and Esko, 1994) while biosynthesis of CS/DS occurs when numbers of acidic amino acid residues, surrounding the ser-gly sequences are reduced (Esko and Zhang, 1996). However, none of the described sequences explains the

mechanism that determines whether a protein is turned into a heparin/HSPG or into another type of PG as the same core protein may carry either HS or CS in various tissues.

Addition of the first GlcNAc is believed to be catalyzed by EXTL (Exostosin Like) enzymes which are member of the exostosin (EXT) enzyme family (Kim et al., 2001, Zak et al., 2002, Sugahara and Kitagawa 2002, Busse et al., 2007). Following the addition of the GlcNAc, polymerisation of HS by alternating additions of GlcA and GlcNAc to the non-reducing end of the chain is carried out by a complex of two other proteins of the EXT-family, EXT1 and EXT2 (Senay et al., 2000, McCormick et al., 2000). (The EXT-family is described more in detail below).

Heparan sulphate chain modification reactions

As the chain polymerizes, it undergoes a series of modification reactions that include N-deacetylation followed by N-sulphation of glucosamine residues, epimerization of GlcA to iduronic acid (IdoA) and finally O-sulphation at various positions (Kjellén and Lindahl 1991, Esko and Lindahl, 2001). The extent of these reactions varies, giving rise to enormous structural heterogeneity of HS chains. The first modification, N-deacetylation followed by N-sulphation of glucosamine units, has a key regulatory role in determining the overall structure and charge density of HS, as N-sulphation of glucosamine units is required for all subsequent modifications. The first modification is catalysed by one or more of the four N-deacetylase/N-sulfotransferase (NDST) isoenzymes (NDST1-4). These enzymes have dual action; removal of N-acetyl groups from GlcNAc residues generating $GlcNH_3^+$ followed by the sulphation of generated free amino groups (Lindahl et al., 1998, Grobe and Esko, 2002). The central role of the NDST enzymes in polymer modifications is due to that substrate recognition requirements of subsequent modification enzymes. Further modifications of HS chains, such as GlcA C5-epimerisation and O-sulphation reactions, are concentrated around the regions of N-sulphated (NS) GlcN residues (Lindahl et al., 1998,

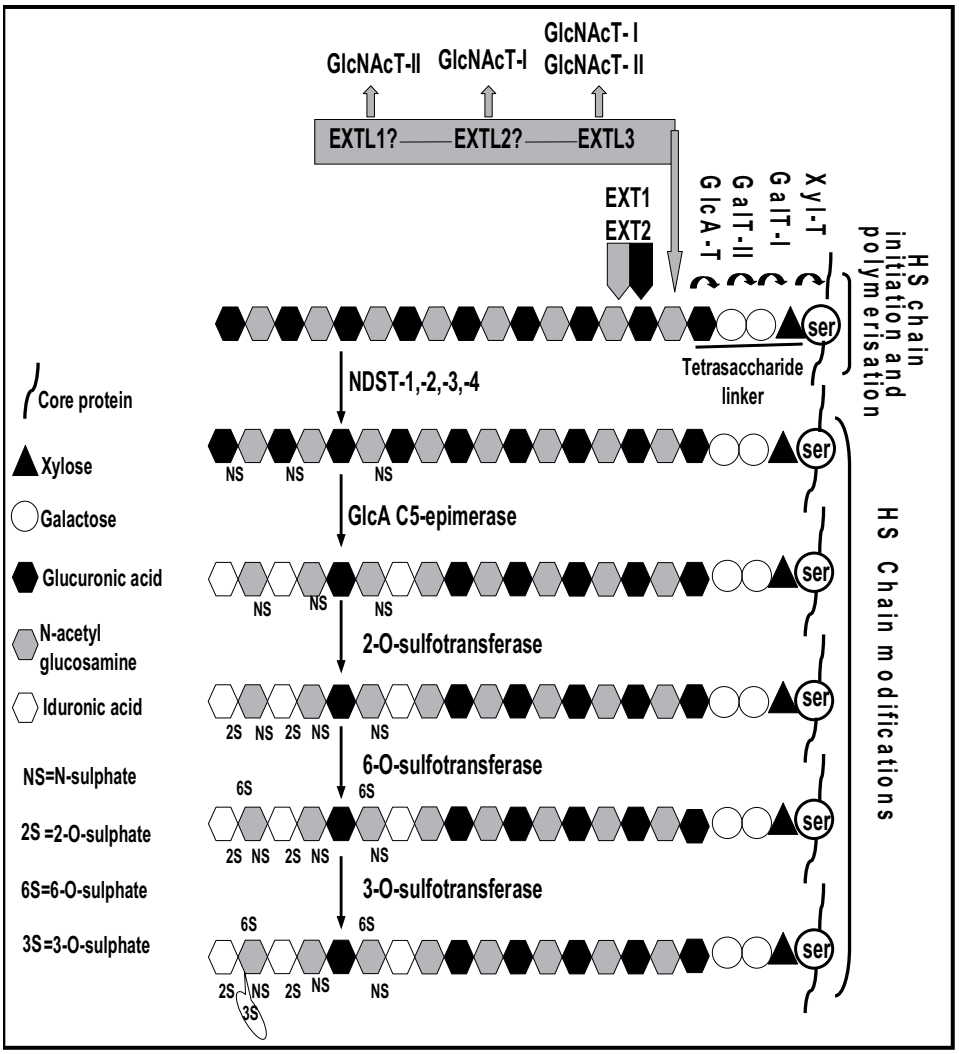


Figure 3. Schematic overview of heparan sulphate biosynthesis.

HS biosynthesis is initiated by addition of xylose to serine residue in core protein. This is followed by the sequentional addition of two galactose residues and a GlcA residue, thus forming the tetrasaccharide linker. The transfer of a single GlcNAc unit to the tetrasaccharide linker is catalyzed by GlcNAc transferase-I, possibly represented by EXTL3. The role EXTL1 and EXTL2 in HS biosynthesis is not clearly defined. Enzyme complex of EXT1 and EXT2 elongates the chain by alternate addition of GlcA and GlcNAc residues. Further enzymatic modifications in HS chains include N-deacetylation and N-sulphation of GlcNAc residues, epimerization of GlcA to IdoA and various O-sulphations. Outside the cells, HS chains are further modified by SULF1 and SULF2 that remove 6-O-sulphate groups from selected glucosamine units (not shown).

Gallagher et al., 2001, Esko and Selleck, 2002). Inactivation of NDST-1 in mice cause severe brain, facial and eye defects (Grobe et al., 2005), together with skeletal abnormalities (Pallerla et al., 2007). The mutant mice die due to respiratory failure (Ringvall et al., 2000, Forsberg and Kjellén, 2001). Mice lacking NDST-2, are fertile with a normal life span and normal HS structure but have defect in connective tissue mast cell morphology and lack sulphated heparin (Forsberg et al., 1999, Ledin et al., 2004). Epimerisation of GlcA into IdoA is catalysed by the enzyme glucuronyl C5-epimerase (Jacobsson et al., 1984, Li et al., 1997). Inactivation of epimerase results in synthesis of HS containing GlcA but no IdoA. Epimerase mutant mice show renal agenesis, skeletal malformations and immature lungs and the mutants die from respiratory failure (Li et al., 2003). The 2-O-sulfotransferase (2OST) transfers sulphate to both IdoA and GlcA residues, with a strong preference for sulphate transfer to IdoA (Rong et al., 2000, Rong et al., 2001). 2OST deficient mice show renal agenesis and die in the neonatal period. These mice also have eye and skeletal abnormalities (Bullock et al., 1998, Forsberg and Kjellén, 2001). The HS chains from 2OST deficient cells have large NS domains containing IdoA with no 2-O sulphation but with increased 6-O sulphation. Except for the presence of IdoA, these chains structurally resemble the HS from the C5-epimerase deficient mice, suggesting that 2-O-sulphation of IdoA is necessary for proper kidney development (Kamimura et al., 2001, Merry et al., 2001). 6-O-sulphation and 3-O-sulphation of glucosamine units are performed by the respective O-sulfotransferases (OSTs) (Liu et al., 1999, Smeds et al., 2003). Three HS 6-O-sulfotransferas (6OST1-3) are known (Habuchi et al., 2003). Disruption of the single 6OST in *D. Melanogaster* is embryonic lethal (Kamimura et al., 2001). Mice lacking 6OST-1 have defects in control of axon movements indicating that 6OST-1 has an important role in mammalian axons development (Pratt et al., 2006). 6OST-2 knock out embryos show abnormal muscle phenotype (Bink et al., 2003). HS 3-O-sulphation of glucosamine units is the last modification reaction carried out by 3OSTs (Liu et al., 1999). 3OSTs have seven isoforms in mammals (3OST1, 2, 3A, 3B, 4, 5 and 6) (Shworak et al., 1999, Xia et al., 2002). Heparin is used clinically as an anticoagulant and this activity is mediated by

the binding of a unique pentasaccharide sequence in heparin to the protease inhibitor antithrombin. 3-O-sulphation is essential for the interaction of heparin and HS with antithrombin, however mice lacking 3-OST-1 do not show a procoagulant phenotype despite reduction in level of antithrombin binding HS (HajMohammadi et al., 2003).

Once the synthesis of HSPG is completed it is transported from the Golgi apparatus to ECM or to the cell surface (Bishop et al., 2007). At the cell surface, the HS chains are further modified by HS 6-O-endosulfatases (SULFs: SULF1 and SULF2). SULFs are associated with the plasma membrane and modify HS chain by removing 6-O-sulphate groups from glucosamine units (Ai et al., 2003, Ai et al., 2006). Removal of 6-O-sulphates has a major effect on HS-binding ligands and cell signalling pathways (Dhoot et al., 2001, Ai et al., 2003). For example, fibroblast growth factor (FGF) signalling requires HS-6-O-sulphate groups to form a functional HS/FGF/FGF-receptor signalling complex (Schlessinger et al., 2000). SULF1 or SULF2 mutant mice appear normal but double mutant mice show delayed myogenic differentiation and regeneration (Langsdorf et al., 2007). Each cell in our body synthesises HS chains of different structure and of different length. The molecular diversity of HS is due to the incomplete action of HS enzymes involved in the HS modification reactions and remodelling of HS sulphation pattern at the cell surface by SULFs (Dhoot et al., 2001, Ai et al., 2003).

The mature HS chain is heterogeneous in nature and is composed of three different domain structures, these are unmodified non-sulphated N-acetylated (NA) regions with GlcA residues, contiguous NS regions that are 2-O- and 6-O-sulphated and contain both GlcA and IdoA units, and mixed sequences of alternating NS and NA disaccharide units. The mixed sequences, like the NS domains, contain GlcA, IdoA and 6-O-sulphated residues, but lack 2-O-sulphate groups (Turnbull and Gallagher 1991, Maccarana et al., 1996, Murphy et al., 2004).

THE EXOSTOSIN FAMILY

There are five members of the exostosin (EXT) family in humans (Table 2); EXT1, EXT2, EXTL1, EXTL2 an EXTL3. All members of the EXT family are type II transmembrane proteins with a short amino terminal cytoplasmic tail, a transmembrane domain, stem region and a large catalytic domain (McCormick et al., 1998, McCormick et al., 2000, Pedersen et al., 2003) (Figure 4). All EXTs contain at least one aspartic acid-any amino acid-aspartic acid (DXD) motif critical for binding UDP-sugars that is shared by most glycosyltransferases (Pedersen et al., 2003). The EXT amino acid sequences are most similar in their C-terminal regions (Nadanaka and Kitagawa, 2008).

EXT1 and EXT2

In vitro studies have shown that EXT1 have both GlcNAc-transferase and GlcA-transferase activity and is able to polymerize HS on oligosaccharide substrates, whereas EXT2 only adds a single GlcA without further elongation (Busse and Kusche-Gullberg 2003, Kim et al., 2003). The physiological active chain elongation unit is generally believed to be a hetero-complex of EXT1/EXT2. Such hetero-complex has increased transferase activities compared to the activities observed for EXT1 or EXT2 alone (McCormick et al. 2000, Senay et al. 2000). EXT1 and EXT2 mutants fail to undergo gastrulation and are embryonic lethal (Lin et al., 2000, Lin et al., 2004, Stickens et al., 2005). siRNA silencing of EXT1 or EXT2 results in synthesis of shorter HS chains (Busse et al., 2007). EXT genes are well conserved between human, *D. melanogaster* and *C. elegans* and are essential in the biosynthesis of HS (Clines et al., 1997, Bellaiche et al., 1998). ttv (tout-velu) is the ortholog of EXT1 and sotv (sister of tout-velu) is the ortholog of EXT2 in *D. melanogaster* (Bellaiche et al., 1998). Mutations in sotv and ttv lead to defective HS biosynthesis, impaired hadgehog, wingless, Decapentaplegic signalling and morphogen distribution

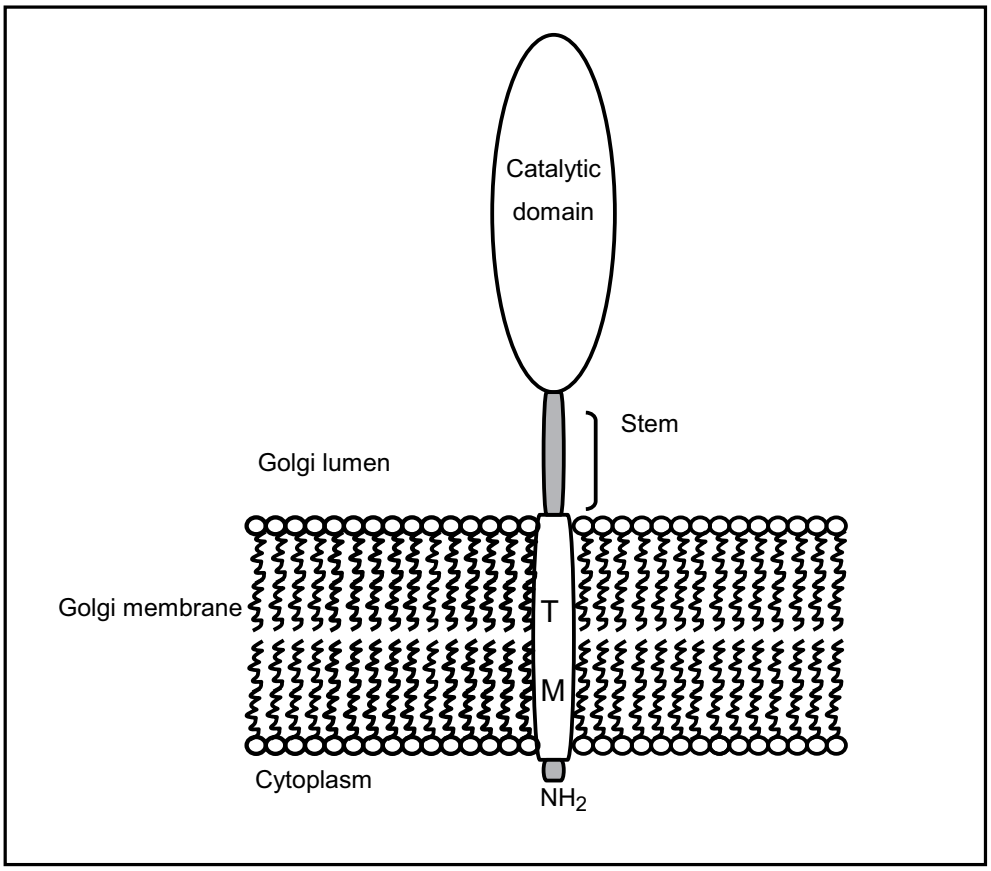


Figure 4. Typical topology of type II transmembrane glycosyltransferases.

Golgi glycosyltransferases and sulfotransferases generally have a single hydrophobic transmembrane (TM) domain flanked by a short amino-terminal domain and a large catalytic C-terminal region. This topology places the catalytic domain of a glycosyltransferase within the lumen of the Golgi apparatus.

(Bellaiche et al, 1998, Takei et al., 2004) (Table 2). rib-1 and rib-2 are homologous to mammalian EXT1 and EXTL3 respectively in *C. elegans* (Clines et al., 1997). rib-1 is required for HS biosynthesis and during embryonic morphogenesis in *C. elegans*. rib-1 knockout mutants show reduction in HS biosynthesis and embryonic lethality (Kitagawa et al., 2007). Coexpression of rib-1 and rib-2 results in increased glycosyltransferase and polymerase activities (Kitagawa et al., 2007).

Table 2. Properties of five human EXTs

Protein	Molecular weight kD	Number of amino acid	Chromosome localisation	Expression pattern	Reference
EXT1	86	746	Chr.8 q24.11	ubiquitous	Cook et al., 1993
EXT2	82	718	Chr.11 p11.2	ubiquitous	Wu et al., 1994 Wuys et al., 1995
EXTL1	75	676	Chr.1 p36.11	brain, liver, muscles	Wise et al., 1997 Hul et al., 1998
EXTL2	38	330	Chr.1 p21.2	ubiquitous	Kitagawa et al., 1999
EXTL3	105	919	Chr.8 p21.1	ubiquitous	Wise et al., 1997 Hul et al., 1998

(Table modified from Hul et al., 1998)

EXTL1

EXTL1 is more closely related to EXT1 than the other EXT members (Freeman and Gurdon, 2002). EXTL1 is found in all investigated mammals but not in lower vertebrates. Human EXTL1 is expressed in limited areas such as skeletal muscles, brain and heart (Wise et al., 1997). In adult mouse, EXTL1 is strongly expressed in liver, skeletal muscles, brain and heart (Stickens et al., 2000). The more restricted expression of EXTL1 may suggest a more specialized function. It has been shown by *in vitro* studies that EXTL1 adds GlcNAc to the non-reducing end of growing HS chain (GlcNAc transferase-II activity) (Kim et al. 2001, Kim et al. 2002, Kim et al., 2003). No mutants of EXTL1 are described yet. Precise role of EXTL1 in the regulation of HS chain is not known.

EXTL2

Similar to EXTL1, EXTL2 is found in all investigated mammals but not in lower vertebrates. EXTL2 is the shortest member of the EXT family consisting of 330

amino acids (Pedersen et al., 2003). EXTL2 is located at chromosome 1p21 which is the region involved in many chromosomal rearrangements in many tumours (Wuyts et al., 1997). EXTL2 is ubiquitously expressed in all human tissues. Phylogenetic analyses of EXT gene family based on amino acid sequences alignments show that EXTL2 is the most divergent, indicating that it was the first gene to evolve separately from the other EXT family members from a common ancestor (Hul et al., 1998). EXTL2 has shown to have GlcNAc-TI activity *in vitro* i.e. adds the first GlcNAc unit to the GlcA in the polysaccharide protein-linkage region (GlcA-Gal-Gal-Xyl-O-Ser) (Kim et al., 2001, Sugahara and Kitagawa, 2002). EXTL2 also transfers N-acetylgalactosamine to the linkage region *in vitro* (Kitagawa et al., 1999, Pedersen et al., 2003). Mouse EXTL2 is the only member of EXT gene family to be crystallized as ternary complex with UDP and the acceptor substrate (Pedersen et al., 2003). The structure shows three highly conserved residues in EXTL2 which are located at the active binding site of EXTL2 and play important roles in glycosyltransferase activity of catalytic globular domain by correctly positioning the substrate for the binding. This domain catalyzes both UDP-GalNAc and UDP-GlcNAc transferase activities and mutations in this region reduce the EXTL2 catalytic activity. The two subdomains of the catalytic domains, described as UDP-sugar- binding domain and acceptor binding domain are connected with loop and contain the conserved DXD motif. DXD motif provides the main binding element for catalytic metal Mn^{+2} ions and UDP-sugar donor and helps in keeping them into correct place and orientation and coordinates for catalytic activity by inducing conformational changes in the active binding site. (Pedersen et al., 2003, Negishi et al., 2003). However, the primary function of EXTL2 *in vivo* is not clear yet.

EXTL3

In contrast to the other two EXTLs, EXTL3 is expressed in all investigated species from sea anemones to humans. EXTL3 is ubiquitously expressed in human tissues in both embryonic and adult lives (Wise et al., 1997, Saito et al., 1998, Litwack et al.,

1998). EXTL3 has a role in brain development and corticogensis in mouse embryo, and in adult mice in the apical dendrite of pyramidal neurons (Litwack et al., 1998, Osman et al., 2004). *In vitro* analyses indicate that EXTL3 has both GlcNAc transferase-I and GlcNAc transferase-II activities and thus may be involved in the initiation and/or elongation of HS chain (Kim et al., 2001, Kim et al. 2002, Kim et al. 2003, Takahashi et al., 2009). Human EXTL3, rib-2 and botv (brother of tout-velu, EXTL3 ortholog in *D. Melanogaster*) share high amino acid sequence homology especially in C-terminal parts. rib-2 has, similar to the human counterpart, both GlcNAc transferase-I and transferase-II activities. rib-2 mutants show developmental delay and egg laying defects due to defective HS biosynthesis (Kitagawa et al ., 2001, Morio et al., 2003, Nadanaka and Kitagawa, 2008). HS levels are reduced in botv mutants and this results in severely impaired signalling (Tumova et al., 2000, Kim et al., 2001, Bornemann et al., 2004, Takei et al., 2004).

EXTL3 has been suggested to be the receptor for pancreatic β-cell regenerating (Reg) protein in humans and in rats (Kobayashi et al., 2000, Okamoto and Takasawa, 2002). β-cell specific EXTL3 knock out mice are embryonic lethal and die around embryonic day 9 and lack HS-derived disaccharide confirming the loss of EXTL3 in β-cell results in marked defective HS biosynthesis. Thus, EXTL3 appears to be essential for HS biosynthesis in these β-cell specific EXTL3 knock out mice and the other EXT members can not compensate for this loss of EXTL3 (Takahashi et al., 2009). Previous results from our lab indicate that the cells transfected with siRNA against EXTL3 have longer HS chains as compared to control chains and it was speculated that siRNA treatment of EXTL3 results in the synthesis of fewer linkage region containing the first GlcNAc for elongation of HS chain and thus there is more extensive polymerisation of the HS chains that are synthesized (Busse et al., 2007). In a recent paper by Okada and colleagues it was shown that knockout of EXTL3 in EXT1 defective cells had little effect on the biosynthesis of HS (Okada et al., 2010). In the paper it was speculated that EXTL3 may have a role in regulating the number and length of HS chains and not be necessary for HS chain elongation (Okada et al., 2010).

HEREDITARY MULTIPLE OSTEOCHONDROMA

Hereditary Multiple Osteochondromas (MO) or Hereditary Multiple Exostoses is an autosomal dominant human disorder linked to mutations in EXT1 and EXT2 (Wuyts and Hul, 2000). MO affects 1:50 000 of the population and usually develops before the age of 10 (Schmale et al., 1994). MO is characterized by cartilage capped bony outgrowths at the end of long bones (Solomon 1963, Ahn et al., 1995, Wuyts et al., 1996). Common complications are short stature and skeletal deformities (Solomon et al., 1963, Hennekam 1991, Stieber and Dormans, 2005). There are 1-2% risks of malignant transformation of MO into chondrosarcoma (Solomon et al., 1963, Schmale et al., 1994). Endochondral bone growth is a strictly regulated process, taking place in the growth plate in which proliferating chondrocytes pass different stages of proliferation and differentiation until they constitute a scaffold for invading osteoblasts. Histologically the chondrocytes are discernable in columnar structures with the direction of differentiation towards the diaphysis. Chondrocyte morphology of an exostose resembles growth plate columnar histology, but is highly disorganised and HS production is severely reduced (Hecht et al., 2002). In the growth plate, HS is involved in regulation of chondrocyte differentiation and proliferation through Indian Hedgehog/parathyroid hormone like hormone and FGF signalling (Vortkamp et al., 1996, Cormier et al., 2002). One theory on the aetiology of MO is that mutations in EXT1 or EXT2 result in decreased HS formation which reduces Indian Hedgehog/parathyroid hormone like hormone and FGF signalling. This results in abnormal chondrocyte proliferation leading to exostoses (Kroneberg 2003, Koziel et al., 2004). Mutations in EXTLs have so far not been linked to MO (Kitagawa et al., 1999).

HEPARAN SULPHATE FUNCTIONS

HS chains bind to a large number of ligands such as growth factors and their receptors, morphogens, plasma proteins and basement membrane components

(Rapraeger et al., 1991, Huntington 2003, Bishop et al., 2007). The binding of HS to proteins has important physiological functions by influencing many cellular processes such as embryonic development, morphogen distribution, intracellular signalling, angiogenesis and cell adhesion (Figure 5) (Huntington 2003, Kramer and Yost 2003,

Table 3. Glycosyltransferase activities of EXTs

Protein	GlcNAcT-I	GlcNAcT-II	GlcAT-1	Phenotype	References
rib-1	-	-	-	Developmental defects in embryo	Kitagawa et al., 2007
ttv	-	+	+	Defect in Hh, Dpp signalling	Bellaiche et., 1998
mEXT1	-	+	+	Disruption of gastrulation	Lin et al., 2000
hEXT1	-	+	+	Exostoses	McCormick et al., 2000
sotv	-	+	+	Defect in Hh and Dpp signalling	Han and Takei et al., 2004
mEXT2	-	+	+	Disruption of gastrulation	Stickens et al., 2005
hEXT2	-	+	+	Exostoses	McCormick et al., 2000
mEXTL1	NT	NT	NT	?	
hEXTL1	-	+	-	?	Kim et al., 2001
mEXTL2	+	-	-	?	Kitagawa et al., 1999
hEXTL2	+	-	-	?	Kitagawa et al., 1999
rib-2	+	+	-	Developmental defects in embryo	Kitagawa et al., 2001
botv	+	+	-	Defect in Hh, Dpp signalling	Han et al., 2004
mEXTL3	NT	NT	NT	Embryonic lethal at E10.5	Takahashi et al., 2009
hEXTL3	+	+	-	?	Kim et al., 2001

(Table modified from Nadanaka and Kitagawa, 2008)

- = no activity NT = not tested m=mouse h=human Hh= Hedgehog Dpp=Decapetaplegic
botv=brother of tout-velu, EXTL3 ortholog in *D. melanogaster*
rib-1 and rib-2= EXT1 and EXTL3 ortholog in *C.*
sotv= sister of tout-velu, EXT2 ortholog in *D. melanogaster*

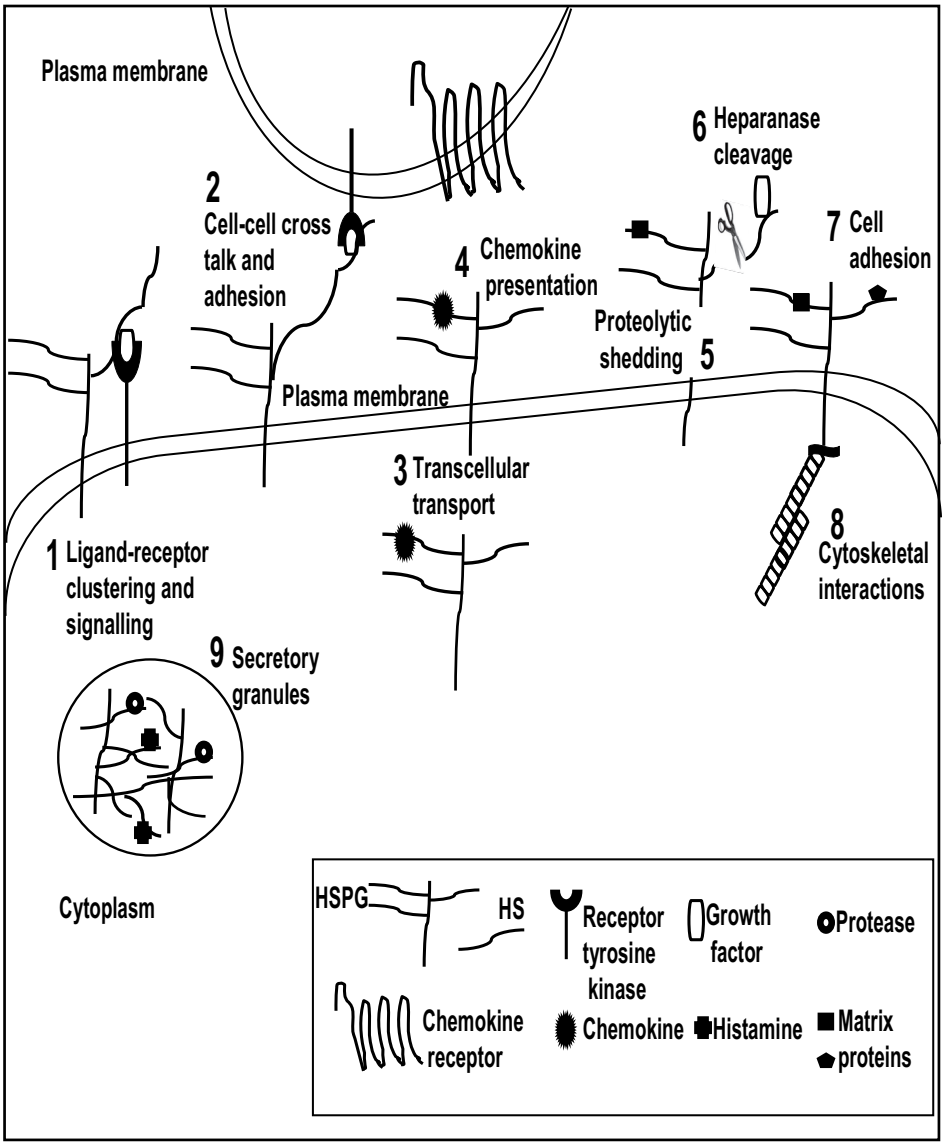
Selleck 2006, Bishop et al., 2007, Kirkpatrick and Selleck, 2007). HSPGs biological functions usually involve one or more HS chains. The linear and sulphate substituted HS chains contribute to selective protein binding through highly specific saccharide sequence motifs in the HS chains (Spillman and Lindahl 1994, Turnbull et al., 2001). HS can modulate protein function in many different ways. The mechanisms by which HS modulates the signalling activity of several growth factors include growth factor receptor dimerizations, receptor ligand complex stabilisation, reducing the dimensionality and cellular transport of ligands. In dimensional sliding contact, HS chain reduce the dimensionality of the molecule and allow more frequent encounter with the high affinity signalling receptor molecules which are present in less quantities and can trigger a biological response by forming complexes with the

molecules (Baeg et al., 2001). In transcellular mechanism, molecules reach remotely placed cells by moving through neighbouring cells using HS (Nybakken and Perrimon, 2002). One of the most studied HS ligand is FGF-2. FGF is a family of secreted proteins produced by variety of cells and is important during organogenesis and in adult life (Ornitz and Itoh, 2001). Binding of FGFs to their tyrosine kinase signalling receptors (FGFRs) requires HS (Rapraeger et al., 1991, Nurcombe et al., 1993, Schlessinger et al., 2000). The FGF-FGF-receptor complex stabilizes and protects FGF molecules against inactivation and results in prolonged transmembrane signalling and biological response (Nurcombe et al., 1993).

The plasma proteinase inhibitor antithrombin inhibits serine proteases involved in blood coagulation (Bjork and Lindahl 1982, Dahlback, 2000). In presence of heparin, the rate of inactivation is greatly increased (Rosenberg et al., 1977, Rostand and Esko, 1997). This is due to that heparin binding induces a conformational change in antithrombin. High affinity for antithrombin is mediated by a specific pentasaccharide sequence in heparin (Lindahl et al., 1980, Lindahl et al., 1984).

Lipoprotein lipase mediated binding of lipoprotein to HS serves an important biological function. HS binding at cell surface and in the ECM brings lipoprotein close to cell surface to facilitate its metabolic processes that occur at the cell surface (Eisenberg et al., 1992). Liver HS can act as a triglyceride rich lipoproteins clearance receptor. Syndecan-1 facilitates lipoprotein uptake and degradation in liver hepatocytes (Fuki et al., 1997, Mahley and Ji 1999, Bishop et al., 2008, Stanford et al., 2009). Syndecan-1 is the primary hepatocyte HSPG receptor in mice which mediates the clearance of both intestinal and hepatic derived triglyceride rich lipoproteins *in vivo*. Syndecan-1 knock out mice show delayed triglyceride clearance from dietary fat and accumulate triglyceride rich lipoproteins (Stanford et al., 2009).

HS chains can act as coreceptor for herpes simplex virus and Neisseria gonorrhoeae (Rostand and Esko 1997, Dehioa et al., 1998). Removal of HS at cell



(Figure modified from Bishop et al., 2007)

Figure 5. Heparan sulphate proteoglycans function.

(1, 2) HS presents growth factors to their receptor on the same cell or adjacent cell to form signalling complex. (3, 4) HS may transport chemokines across the cell and presents at the cell surface. (5) Proteolytic shed and (6) heparanase cleavage of HS chains can release HS bound ligands. (7) HS facilitates cell-adhesion and (8) forms bridges to the cytoskeleton. (9) Serglycin carrying heparin chains is required for storage of protease and histamine in secretory granules of mast cells.

surface reduces the invasion of the pathogens (Sawitzky et al., 1996, Summerford et al., 1998).

Cancer is associated with changes in cell morphology and loss of differentiation and uncontrolled proliferation of cells followed by tissue invasion and metastasis (Cho and Fearon, 1995). Many of these processes involve HS. Syndecan-1 is one important determinant of cellular differentiation and has an important role in maintaining normal cell morphology (Mali et al., 1994, Kato et al., 1995, Sanderson and Borset, 2002). Transformation of epithelial cells is associated with loss of syndecan-1 expression in many poorly differentiated carcinomas of head, neck, lung and cervix (Inki et al., 1994, Inki et al., 1996). Syndecan-1 shedding from the myeloma cells can induce apoptosis of the myeloma cells *in vitro* and thus decrease the invasiveness of the tumour (Dhodapkar et al., 1998). Glypican-1 expression is upregulated in pancreatic tumours (Kleef et al., 1998). Glypican-3 shows a very distinct expression pattern in tumourigenesis and can act as negative regulator of cell proliferations by inducing apoptosis and thus inhibit cell proliferation in certain type of tumour cells (Blackhall et al., 2001, Capurro et al., 2005). Glypican-3 is not normally expressed in liver and colon but is upregulated in hepatocellular carcinoma and in colorectal cancer (Filmus 2008, Midorikawa et al., 2003, Ligato et al., 2008). It is worth to note that if the origin of the tumour is from a tissue that expresses glypican-3 only during the embryonic period, glypican-3 expression reappear during malignant transformation. If the origin of cancer is from glypican-3 positive tissue in adult, its expression is reduced in the tumour, which makes the tumour more invasive in nature (Filmus, 2008).

METHODS

Glycosyltransferase assay

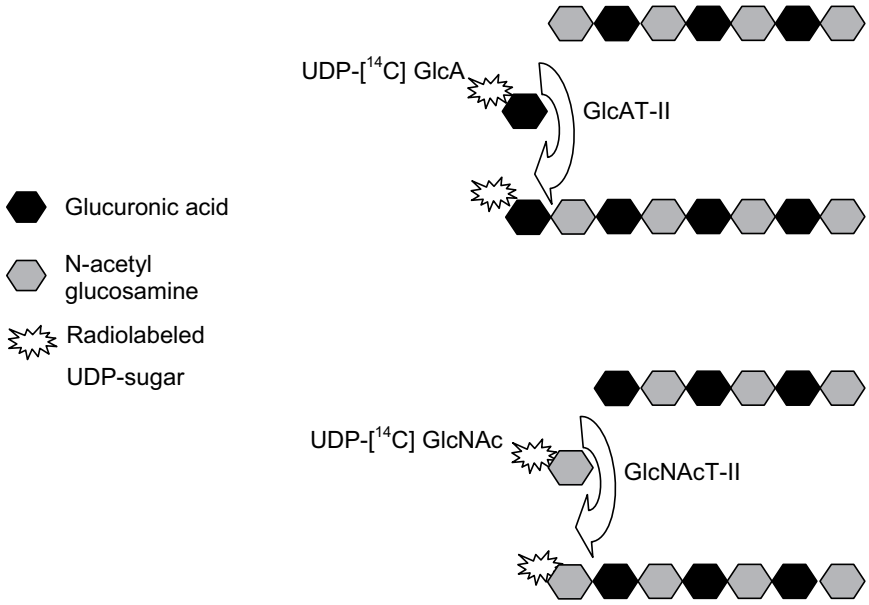


Figure 6. The GlcA-TII and GlcNAc-TII reactions.

The GlcA-TII assay measures the transfer of ^{14}C GlcA unit from the corresponding UDP-sugar to the oligosaccharide acceptor (upper reactions) with a nonreducing end GlcNAc. The GlcNAc-TII assay instead measures the transfer of ^{14}C GlcNAc unit from the corresponding UDP-sugar to oligosaccharide acceptor (lower reactions) with a non-reducing end GlcA.

PRESENT INVESTIGATIONS

AIM OF THE STUDY

The overall aim of this thesis was to study the role of EXTL1, EXTL2 and EXTL3 in HS biosynthesis. The thesis work has been divided into two subprojects; one dealing with EXTL2 (Paper I) and the other with EXTL1 and EXTL3 (Paper II).

RESULTS

Paper I

The role of EXTL2 in heparan sulphate chain elongation

In order to investigate if EXTL2 may have a function in mammalian HS biosynthesis, the effect of reduced or increased levels of human EXTL2 on HS chain elongation was analysed. Human embryonic kidney (HEK) 293 cells were transiently transfected with siRNA targeting EXTL2 in order to suppress EXTL2 expression or cells were transfected with a plasmid containing the full-length EXTL2 to increase its expression level. Downregulation of EXTL2 by siRNA resulted in an increased HS chain length, whereas cells overexpressing EXTL2 produced shorter HS chains as compared to untreated control cells. In addition, EXTL2 was able to catalyze the addition of a single GlcNAc to $[GlcA\text{-}GlcNAc]_n$ oligosaccharide acceptors (GlcNAc-TII activity), but did not show any detectable transfer of GlcA to $GlcNAc[GlcA\text{-}GlcNAc]_n$ oligosaccharide acceptors (GlcA-TII activity).

Paper II

Effect of overexpression of EXTL1 on heparan sulphate chain length

In this paper, HEK 293 cells were stably transfected with EXTL1 and EXTL3 and the effect of overexpression on HS chain elongation was studied. Overexpression of either EXTL1 or EXTL3 resulted increased chain length and an altered disaccharide composition. Cells overexpressing EXTL1 or EXTL3 produced HS chains with

decrease in IdoA2S-GlcNS and increase in GlcA-GlcNS6S disaccharide unit. To study the effect of down regulation of EXTL3 on HS chain elongation, HEK 293 cells were transiently transfected with siRNA targeting EXTL3. Similar to cells overexpressing EXTL3, cells silenced with siEXTL3 also synthesized longer HS chains. In addition, EXTL1 and EXTL3, showed identical *in vitro* enzyme activities with each other and with EXTL2 (paper I). They catalyzed the addition of a single GlcNAc to $[GlcA\text{-}GlcNAc]_n$ oligosaccharide acceptors (GlcNAc-TII activity), but showed no detectable transfer of GlcA to $GlcNAc[GlcA\text{-}GlcNAc]_n$ oligosaccharide acceptors (GlcA-TII activity).

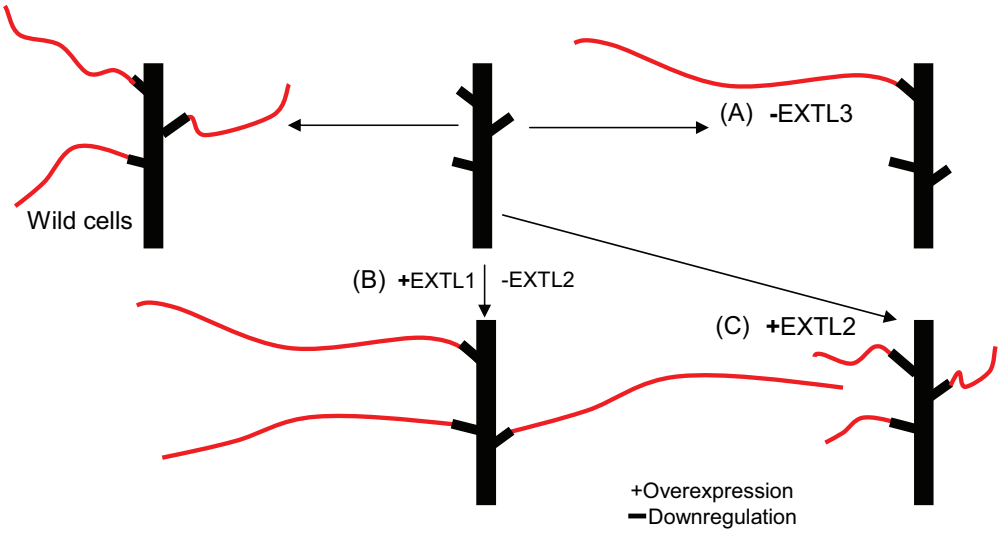


Figure 7. Effects of up or down regulation of EXTL1, EXTL2 or EXTL3 on heparan sulphate chain elongation.

(A) Reduction in EXTL3 results in synthesis of longer HS chains. (B) Gene silencing of EXTL2 and overexpression of EXTL1 and EXTL3 produces HS chain that are longer than wild type cells. (C) EXTL2 overexpression results in shorter HS chains.

DISCUSSION

The mechanism that regulates the molecular diversity of HS is not fully understood and in particular we lack knowledge about the regulation and topography of the biosynthetic machinery involved in HS biosynthesis. HS has a unique molecular design and is a selective binding partner of several different proteins and is involved in many biological processes including embryonic development, differentiation, morphogen distribution and cell signalling. HS biosynthesis, which occurs in the Golgi apparatus, is a complex process involving many enzymes and enzyme isoforms. This process generates a non-sulphated polysaccharide chain that is modified by a sequential and coordinated series of enzyme reactions to form the mature HS chain. To understand HS-related biological functions that are dependent on the structural variability of HS we need to understand the function and regulation of enzymes involved in HS biosynthesis. HSPGs are an important feature of the cell surface and ECM. Both HS fine structure and chain length play important roles in determining the binding site for HS-dependent signalling factors and thus influence HS interaction with a variety of molecules (Lindahl et al., 1998, Österholm et al., 2009). The length of HS chains varies. Most chains contains between 50-200 disaccharide units (25-100 kDa), and due to that HS chains form extended helical coil structures, this size range corresponds to 40-160 nm in length (Turnbull et al., 2001). The mechanism controlling HS chain elongation and chain termination is poorly understood. EXT1-EXT2 is a heter-oligomeric complex that is necessary for HS chain elongation (McCormick et al., 2000, Senay et al., 2000, Busse and Kusche-Gullberg, 2003). The three EXTLs (EXTL1, EXTL2, EXTL3) share aminoacid sequence homology with EXT1 and EXT2 (Kitagawa et al., 1999, Kim et al., 2001) indicating that EXTLs could be involved in HS chain biosynthesis but, except for EXTL3, their involvement in HS biosynthesis is unclear. To clarify the respective function of EXTL1, EXTL2 and EXTL3 in HS biosynthesis, the effect of reduced or increased levels of human EXTL1, EXTL2 and EXTL3 proteins in HEK 293 cells on HS chain elongation and HS fine structure was analyzed.

Previous results from our lab have shown that gene silencing of EXTL3 produces longer HS chains (Busse et al., 2007). Our recent results are in consistent with the previous findings. In the paper by Busse et al. (Busse et al., 2007) it was speculated that EXTL3 was responsible for adding the first GlcNAc to the linkage region. With reduced amounts of EXTL3, fewer chains would be initiated but as the number of EXT1/EXT2 complex was not affected, this resulted in more extensive polymerisation (Busse et al., 2007). Mice deficient in EXTL3 die neonatally and lack HS further supporting the idea that EXTL3 is an initiator of HS synthesis (Takahashi et al., 2009). *C. elegans* mutants lacking rib-2 (*C. elegans* ortholog of EXTL3) has reduced production of HS (Morio et al., 2003). Mutation in *box* (the zebrafish ortholog of EXTL3) also shows a dramatic decrease in HS biosynthesis and the mutant phenotype can be rescued by injection of wild type *box* mRNA (Lee et al., 2004). Our recent results, that overexpression of EXTL3 also resulted in longer chains (paper II) complicates the picture and indicates that EXTL3, in addition to initiate the chains, also influences chain elongation. It has been speculated that EXTL3 could be a terminator of chain elongation (Kim et al., 2001) but if this was the case, overexpressing EXTL3 should have resulted in shorter chains. Interestingly, removal of HS chains by heparitinase treatment upregulated Extl3 mRNA levels in mouse pancreatic β-cells, indicating a feed back mechanism where EXTL3 is regulating HS biosynthesis (Takahashi et al., 2009).

EXTL2 has been shown to have only GlcNAc-TI activity and not GlcNAc-TII activity (Kitagawa et al., 2001) but here we show that it has GlcNAc-TII activity as well. This is very interesting and could indicate that EXTL3 and EXTL2 have similar roles in HS synthesis. However, although siRNA of EXTL2 (Paper I), similar to corresponding treatment of EXTL3 (Paper II), resulted in synthesis of longer chains, overexpression of EXTL2, in contrast to similar treatment of EXTL3, resulted in synthesis of shorter chains, indicating that they may have different functions. Furthermore, in agreement with out results, transgenic mice overexpressing EXTL2 have reduced amount of HS (Nadanaka and Kitagawa, 2008). Okada et al. (Okada et al., 2010) speculated that in cells lacking EXT1, HS chains were initiated

by EXTL2 and polymerisation carried out by EXT2 (Okada et al., 2010). Synthesis of longer HS chain after siRNA could indicate that EXTL2 is a chain terminator or an inhibitor of HS elongation. This could also explain the finding that EXTL2 overexpression results in shorter HS chains. siRNA of EXTL2 also resulted in increased HS/CS ratio but did not seem to influence the HS disaccharide composition. The changes in HS/CS ratio was observed both for ^{35}S- and ^{3}H-labelled material (Paper II and unpublished data, respectively) and could possibly indicate that there is a limited amount of UDP-GlcA in the Golgi lumen. With increased synthesis of HS the UDP-GlcA pool may be insufficient for both HS and CS synthesis. One important point to have in mind is that our values relate to cell surface/ECM associated PGs and may not reflect the total GAG production.

Cells overexpressing EXTL1 produced longer chains with altered disaccharide structure. Increased levels of EXTL1 may have resulted in increase incorporation of GlcNAc residue on the growing HS chain and thus resulting in longer HS chains. As chain elongation in HS involves the action of EXT1 and EXT2 to catalyze the transfer of GlcA and GlcNAc from their respective UDP-derivatives to the non-reducing end of growing polymer (Busse and Kusche-Gullberg, 2003) it may be speculated that EXTL1 can form a complex with EXT1 or EXT2 to perform this action. In this case, GlcA will be incorporated by EXT1 and/or EXT2. So a possibility of several different types of multimeric complex involved in HS chain elongation may exist. It has been questioned if EXTL1, because of its restricted tissue specific expression, is involved in HS chain elongation. Our results show, for the first time, that EXTL1 exhibited a significant GlcNAc-TII activity. In addition, increased chain length and an altered disaccharide composition after overexpression indicate that EXTL1 is involved in HS chain elongation. Because HEK 293 cells do not express EXTL1, we could not study the effect of gene silencing of EXTL1 on HS chain length. At the moment we do not know of any human cell line that expresses EXTL1. However, the mouse C2C12 myoblast cell line has been shown to dramatically upregulate Extl1 expression during C2C12 differentiation into myotubes (Janot et al.,

2009). It would be highly interesting to study the effect on HS synthesis after down-regulation of EXTL1 in differentiated C2C12 cells.

Most if not all of the biosynthetic enzymes involved in HS biosynthesis have been cloned. However, we still know little about the organization of the biosynthetic apparatus or the localization of the enzymes in the Golgi membrane. In addition, very little is known about how the biosynthetic enzymes interact with each other and with other proteins. The hypothetic "GAGosome" is a model to explain how the enzymes in the HS biosynthetic machinery cooperate and influence each other. The GAGosome is defined as a complex of different HS enzymes/or enzyme isoforms. In the GAGosome model, the relative amounts of each enzyme/enzyme isoform present in the GAGosome will have an impact on the HS structure. Complexes between different HS enzymes have been described, such as the EXT1/EXT2 complex (Kobayashi et al., 2000, McCormick et al., 2000, Senay et al., 2000) and between the 2OST and the C5-epimerase (Pinhal et al., 2001, Smeds et al., 2010). In a recent study, the authors speculate that the combined N-deacetylase/N-sulfotransferase-1 (NDST1) and EXT1 compete for binding to EXT2, which acts as a transport protein for both enzymes from the endoplasmic reticulum to the Golgi (Presto et al., 2008). Our results in paper I and II, indicate that the at least EXTL1 and EXTL3 may assist EXT1 and/or EXT2 in the polymerization process *in vivo*, since overexpression of these proteins resulted in longer chains. However, if the EXTLs collaborate or form complexes with other proteins involved in HS biosynthesis is still an open question.

Development of hereditary multiple osteochondromas is caused by mutations in either EXT1 or EXT2 and the resulting reduction, but not total loss, of HS in the chondrocytes of exostoses, suggests that exostoses formation is a result of reduced HS (Stickens et al., 2005). Contradictory reports are present on the cellular origin of the osteochondroma and, although tumour suppressor properties were early ascribed to EXT1 and EXT2, it remains unclear if EXT1 and EXT2 fit the classical two-hit model for tumour suppressor genes, which implies that osteochondroma results from a somatic mutation in the remaining wild-type copy of the gene

(Knudson, 1971). In a recent publication, mice with a clonal homozygous inactivation of Ext1 in chondrocytes developed frequent osteochondromas supporting the two-hit model for osteochondroma development (Jones et al., 2009). There is no evidence that defects in the EXTL genes or any other genes involved in HS biosynthesis result in exostoses formation suggesting that only the inactivation of EXT1 or EXT2 in a small fraction of chondrocytes result in the formation of osteochondromas. It is still an open question how exostoses formation due to defective EXT proteins relates to HS biosynthesis.

In conclusion, our study revealed that HS chain length may be controlled by all three EXTLs and that changes in the EXTLs levels influence HS chain length. Still there are many questions that need to be addressed in future. Is it possible that, not only EXT1 and EXT2 elongate HS chains or does it exist several elongation complexes composed of different combinations of EXT/EXTLs? Is EXTL2 an inhibitor of chain elongation or does it have another function? What determines the length of an HS chain? Generation of mice that are deficient in one or more of the EXTLs can be helpful further to understand the role of EXTLs in HS biosynthesis. However, so far very little information has been generated regarding the individual roles of the EXTs in HS biosynthesis from mice deficient in EXT1, EXT2 or EXTL3. They die at an early embryonic stage and lack HS (Lin et al., 2000, Stickens et al., 2005, Takahashi et al., 2009). The individual contribution of each EXTL could be investigated. For example it would be interesting to see whether EXTLs collaborate with one another or with EXT1 and/or EXT2. Alternatively, EXTLs can also form biological complex like EXT1 and EXT2 to polymerise HS chains.

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